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Durable remission related to CAR-T persistence in R/R B-ALL and long-term persistence potential of prime CAR-T

Li Shiqi,[1,7] Zhang Jiasi,[2,7] Chen Lvzhe,[1] Xu Huailong,[3] He Liping,[4] Liu Lin,[1] Zhang Qianzhen,[3] Yuan Zhongtao,[1] Shen Junjie,[3] Chen Zucong,[5] Zhang Yingzi,[3] Wang Meiling,[3] Li Yunyan,[3] Wang Linling,[3] Fang Lihua,[1] Chen Yingnian,[1] Zhu Wei,[3] Li Yu,[1] Luo Le,[1] Wang Youcheng,[1] Zhang Dingsong,[1] Dong Yancheng,[1] Yin Ping,[1] Zhang Lihua,[1] Li Xiaoping,[1] Hu Xiaozhuang,[6] Zheng Zhongzheng,[6] Yang Zhi,[3] Qian Cheng,[3] and Wang Sanbin[1]

[1]Department of Hematology, 920th Hospital of Joint Logistics Support Force of People's Liberation Army of China, Kunming, Yunnan Province 650100, China; [2]Center for Hematology, Southwest Hospital, Army Medical University (Third Military Medical University), Chongqing 400038, China; [3]Chongqing Precision Biotech Co., Ltd., Chongqing 400039, China; [4]Department of Epidemiology and Health Statistics, School of Public Health, Kunming Medical University, Kunming, Yunnan 650500, China; [5]The People's Hospital of Dehong Prefecture, Dehong, Yunnan Province 678400, China; [6]Shanghai Tissuebank Biotechnology Co., Ltd., Shanghai 201318, China

CD19-targeted chimeric antigen receptor T lymphocytes (CAR-T) has demonstrated a high proportion of complete remission in the treatment of relapsed refractory acute B cell lymphoblastic leukemia (r/r B-ALL). It is of great clinical significance to explore which factors will impact long-term disease-free survival of patients with r/r B-ALL after CAR-T therapy without bridging bone marrow transplantation. Our study found that, in patients with r/r B-ALL without bridging transplantation, the patients' age; infusion dosage; whether they had undergone allo-stem cell transplantation before CAR-T therapy, using CD-19-targeted or CD19/CD22-dual-targeted CAR-T; whether there is fusion gene; tumor burden before therapy; and comorbidity had no significant relationship with their long-term disease-free survival. We found only that CAR-T persistence was highly correlated with patients' long-term disease-free survival. So, we further profiled CAR-T cells using single-cell sequencing and found that there is a specific T cell subset that may be associated with the long-term persistence of CAR-T. Finally, according to the single-cell sequencing results, we established cell production process named PrimeCAR, which shared common signaling pathways with the T cell subset identified. In the preliminary clinical study, prime CAR-Ts yield good persistence in peripheral blood of patients with B-ALL and lymphoma, without observing grade 2 or higher cytokine release syndrome.

INTRODUCTION

Chimeric antigen receptor T lymphocytes (CAR-T) targeting CD19 have shown a high response rate in the treatment of acute B cell lymphoblastic leukemia, but relapse after treatment seriously affects the long-term survival of patients.[1–3] The independent clinical trials of Jae H. Park's research team and Kevin A. Hay's research team showed that although CD19-targeted CAR-T therapy can achieve a complete remission (CR) rate of over 80%, the median disease-free survival times are still only 6.1[4] and 7.6[5] months. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) after CAR-T therapy has been suggested to reduce the relapse rate of leukemia.[6–9] Nonetheless, consolidative allo-HSCT is not recommended for all patients because it will increase the economic burden and bring risk of severe toxicity, such as graft-versus-host disease (GVHD), and infections.[10] Therefore, it is of great clinical significance to find some new strategies to reduce recurrence of relapsed refractory acute B cell lymphoblastic leukemia (r/r B-ALL) after CAR-T therapy.

According to the published studies, the relapse after single-targeted CD19 CAR-T therapy can be divided into two subgroups, CD19 expression group and no/weak CD19 expression group.[11–13] Our previous studies have shown that during the treatment of r/r B-ALL with CD19-targeted CAR-T, most of the patients who experienced CAR-T loss within a short time after treatment would relapse, and the relapsed cells are all CD19+.[14] Humanized CAR-T cells have improved *in vivo* persistence, and the disease-free survival of patients has been significantly improved,[15] which is consistent with result reported by Shannon L. Maude et al.[16] This suggests that the persistence of CAR-T may be associated with durable remission. For CD19-related relapse, multi-targeted CAR-T can theoretically address CD19 immune escape of CD19 and have the potential to reduce

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[7]These authors contributed equally

Correspondence: Yang Zhi, Chongqing Precision Biotech Co., Ltd., Chongqing 400039, China.
E-mail: yangzhi@precision-biotech.com
Correspondence: Qian Cheng, Chongqing Precision Biotech Co., Ltd., Chongqing 400039, China.
E-mail: cqian3184@163.com
Correspondence: Wang Sanbin, Department of Hematology, 920th Hospital of Joint Logistics Support Force of People's Liberation Army of China, Kunming, Yunnan 650100, China.
E-mail: sanbin1011@163.com

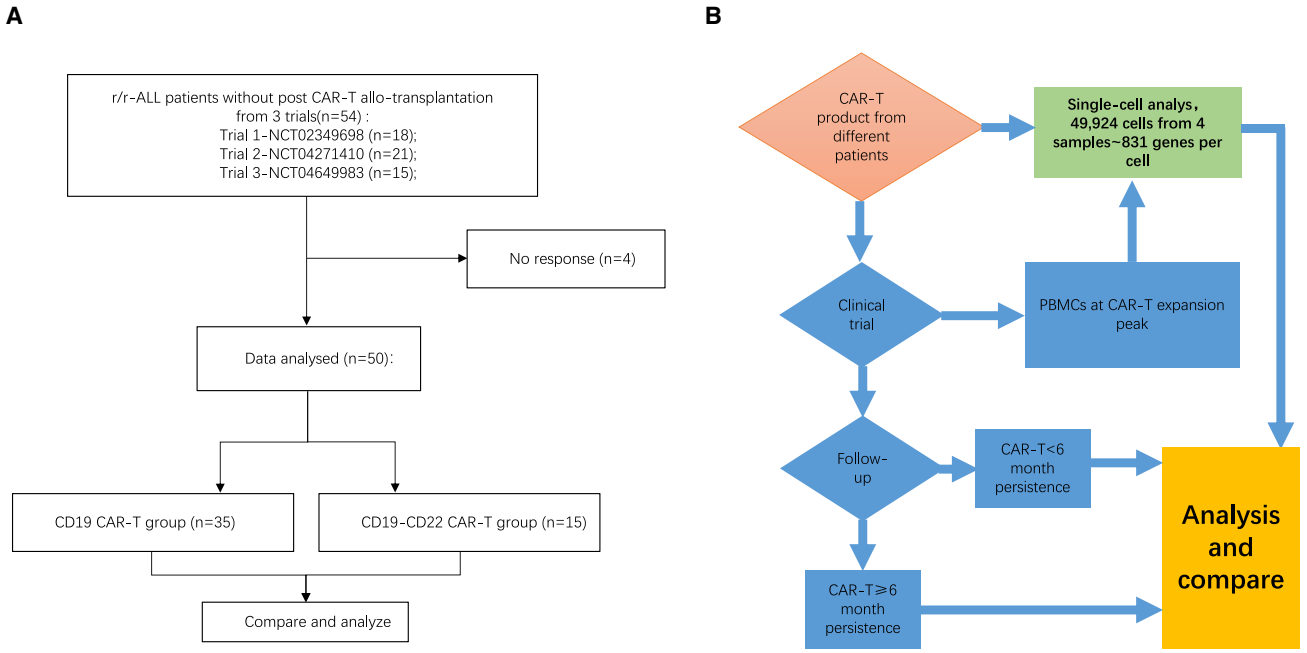


Figure 1. Study workflow

(A) Clinical data inclusion process. (B) Single-cell sequencing analysis workflow. Four samples: one sample is the CAR-T product for one patient with long CAR-T persistence (CAR-long), one sample is the CAR-T product for one patient with short CAR-T persistence (CAR-short), one sample is the PBMCs of peripheral blood at CAR-T expansion peak drawn from the same patient with long CAR-T persistence (PB-long), and one sample is the PBMCs of peripheral blood at CAR-T expansion peak drawn from the same patient with short CAR-T persistence (PB-short).

relapse after treatment. There have been studies trying to use CD22-targeted CAR-T to treat relapsed patients with CD19 immune escape, and there are also studies reporting CD19/CD22-dual-targeted CAR-T to treat r/r B-ALL.[17,18]

Therefore, we analyzed 50 cases of patients with r/r ALL who got a response and no post-CAR transplantation in CAR-T treatment from our clinical study, including 35 cases of CD19-targeted and 15 cases of CD19/CD22-dual-targeted treatment (Figure 1A). After, we determined that the persistence of CAR-T was a core factor affecting the durable remission of patients. We used single-cell sequencing technology to analyze the CAR-T products and CAR-T cells at the peak of amplification time after therapy (Figure 1B), and the patients with long persisting CAR-T and short persisting CAR-T were compared. A group of T cell subsets that may be related to the long-term persistence of CAR-T *in vivo* were found, while the relevant characteristics of their gene expression and transcriptional regulation were preliminarily described.

To extend the *in vivo* persistence of CAR-T cells, we have established the PrimeCAR cell production process by mild activation and shortened *in vitro* amplification time. In preliminary clinical results, prime CAR-Ts made from both CD19-targeting and CD19/CD22 dual-targeting CAR sequences yield good persistence in patients of acute lymphoblastic leukemia and lymphoma, with better safety. It is suggested that PrimeCAR cell production may have the potential to extend the persistence of CAR-T *in vivo*.

RESULTS

Long-term persistence of CAR-T is a key point for durable remission

Univariate risk analysis regarding those 50 patients with r/r B-ALL revealed that the long-term disease-free survival was not significantly related to their age; infusion dosage; whether they had undergone allo-stem cell transplantation before CAR-T therapy; using CD-19-targeted or CD19/CD22-dual-targeted CAR-T; whether there is fusion gene; tumor burden before therapy; or comorbidity (Tables 1 and 2). For the 35 patients treated with CD19-targeting CAR-T, the median follow-up was 6.7 (range: 1.5 to 50.3) months, and the median event-free survival was 8 months (Figure 2A). For the 15 patients treated with CD19/CD22-dual-targeting CAR-T, the median follow-up was 7.6 months (range: 4 to 21), and the median event-free survival was 7.6 months (Figure 2B). There was no significant difference between the two groups in event-free survival ($p = 0.898$).

We set 3 or 6 months as the threshold to define short and long persistence of CAR-T cells. The persistence of CAR-T is defined as a peripheral blood CAR gene copy number ≥ 100 copies/μg genome DNA. In the 35 patients who received CD19 single-target CAR-T cell therapy, 2 patients were followed up with for less than 3 months, and the CAR-T was still detectable. The remaining 33 patients were included in the analysis. Among those, 13 patients achieved ≥ 3 months *in vivo* persistence of CAR-T cells, of which 11 patients achieved ≥ 6 months *in vivo* persistence of CAR-T cells. The median survival time of such

Table 1. Baseline characteristics of the patients treated with ordinary CAR-T

Characteristic	CD19 CAR-T (N = 35)	CD19/CD22 CAR-T (N = 15)
Gender, no.(%)		
Female	22 (63)	12 (80)
Male	13 (37)	3 (20)
Age at infusion, year		
Median	22	32
Range	5–59	7–69
CAR-T dose, ◇10^6/kg		
Median	1.86	2.22
Range	0.08–41.67	0.15–6.32
Allogeneic transplantation history, no. (%)	10 (29)	1 (6)
Baseline burden, no. (%)		
Morphologic remission	20 (57)	9 (60)
5% ≤ marrow blast < 20%	5 (14)	1 (7)
Marrow blast ≥20%	10 (29)	5 (33)
High-risk cytogenetic factors, no.(%)	10 (29)	5 (33)
CNS involvement or extramedullary lesions, no. (%)	10 (29)	5 (33)

Table 2. Single-factor analysis

	Univariate analysis for relapse-free survival	
Variables	HR (95% CI)	p value
Age (24 as cut-off)	1.134 (0.565–2.278)	0.7232
Gender	0.650 (0.296–1.425)	0.2815
Infusion dose (cut-off 3×10^6/kg)	1.811 (0.902–3.638)	0.0952
Transplantation before CAR-T	0.938 (0.405–2.173)	0.8819
Detectable fusion genes	1.230 (0.582–2.600)	0.5885
Tumor burden	1.199 (0.594–2.421)	0.6128
CNS involvement or EMD	0.874 (0.359–2.128)	0.7672

patients could not be determined due to the high survival rate. The event-free survival (EFS) at 24 months was 58% for patients with ≥3 month CAR-T persistence and 70% for patients with ≥6 month CAR-T persistence. Twenty-two patients did not acquire ≥6 month CAR-T persistence, of which 20 patients did not acquire ≥3 month CAR-T persistence. For those with less than 3 months of CAR-T persistence, the median survival time and EFS rate at 24 months were 5.7 months and 13%. For those with less than 6 months of CAR-T persistence, the median survival time and EFS rate at 24 months were 6 months and 12% (Figure 2C). The EFSs between those two groups were both significantly different ($p < 0.001$).

Single-cell analysis reveals the heterogeneity of T cell subsets

We selected 2 patients for in-depth analysis, of which one patient (P-S) had a CAR-T persistence of less than 3 months *in vivo*, while the other patient (P-L) had a CAR-T duration of more than 6 *in vivo* month (Figure 3A). We screened the CAR-T cell products for infusion and the peripheral blood mononuclear cells (PBMCs; at peak of amplification) on day 10 after infusion with single-cell sequencing and obtained 12 T cell subsets (Figure 3B). Analysis on stemness revealed that the top 4 subsets with highest stemness were the 5th, 6th, 7th, and 12th (Figure 3C). The differentiation trajectories of the 12 cell subsets showed that the 4th, 5th, 6th, 7th, 10th, and 12th cell subsets were mostly in the early stages of differentiation (Figure 3D). T cell annotation for the 12 subsets reveal the 3rd, 5th, 6th, 7th, and 12th subsets as CD8+ memory T cells (Figure 3E). Considering the basic immunological concept that T cell persistence is related to memory T cells, the rest of the subsets of memory T cells are unlikely to be related to persistence. Furthermore, in the comparison of content of the 12 subsets among the 4 specimens, the 6th and 7th subsets are significantly more abundant in CAR-T cell products and peripheral blood of the P-L patient than the P-S patient (Figure 3F). Considering the fact that the 12th subset proportion is too small to make a difference and that the uniform manifold approximation and projection (UMAP) graph vectors of RNA transcription rate indicate the earlier stage of differentiation (Figure 3G), we finally consider the 6th and 7th subsets as T cells related to long persistence.

Characteristics of the T cell subgroup with long persistence potential

Based on the above results, we believe that the 6th and 7th T cell subsets are closely associated with the long-term persistence of CAR-T, and we merged those 2 subsets into one group, named T-long. The remaining 10 T cell subsets were merged into another group, named T-other. With those, a new UMAP map was obtained. The proportion of T-long in the CAR-T cell product (CAR-long) and peripheral blood (PB-long) with long persistence was significantly higher than those with short persistence (CAR-short and PB-short) (Figure 4A). The gene expression heatmap and transcriptional regulation analysis demonstrated the difference between T-long and T-other (Figures 4B and 4C). Gene set variation analysis (GSVA) between T-long and T-other subgroups revealed that T-long cell subsets were more active in oxidative phosphorylation, MYC and E2F targets, NOTCH signaling, G2M checkpoint, MTORC1 signaling, glycolysis, fatty acid metabolism, DNA repair, and adipogenesis, indicating the important role of stem cell-related signaling and energy metabolism in T-long (Figure 4D). The distribution of T-long and T-short cells in UMAP is shown in Figure 4E.

Manufacture optimized CAR-T manufactured by optimized method

The features of T-long indicate the importance of early-lineage T cells in maintaining CAR-T persistence. Saba Ghassemi et al. reported reducing CAR-T cell *ex vivo* culture can improve the antileukemic activity and maintain T cell "stemness."[19] According to the finding, we compared several functional parameters at days 1, 2, 3, 7, and 10 of the *ex vivo* expansion and different activation strengths. Finally, we established a cell production platform (named the PrimeCAR

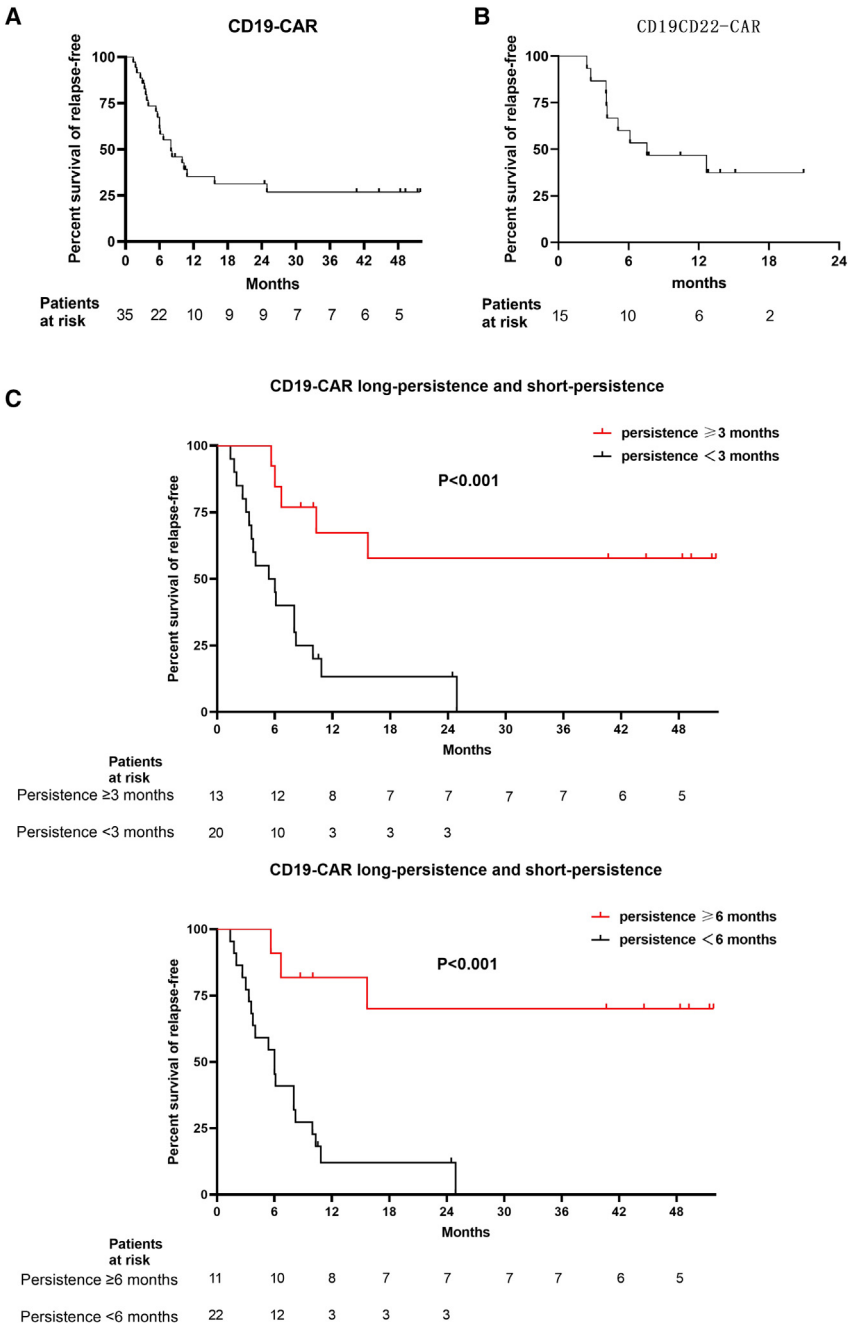


Figure 2. Survival analysis

(A) Relapse-free survival curve of patients with r/r ALL who received CD19-targeted CAR-T cell infusion. (B) Relapse-free survival curve of patients with r/r ALL receiving CD19/CD22-dual-targeted CAR-T cell infusion. (C) Among patients with r/r ALL receiving CD19-targeted CAR-T cell infusion, patients with long-persisted CAR-T cells *in vivo* showed a significant advantage in relapse-free survival than patients with short-persisted CAR-T cells.

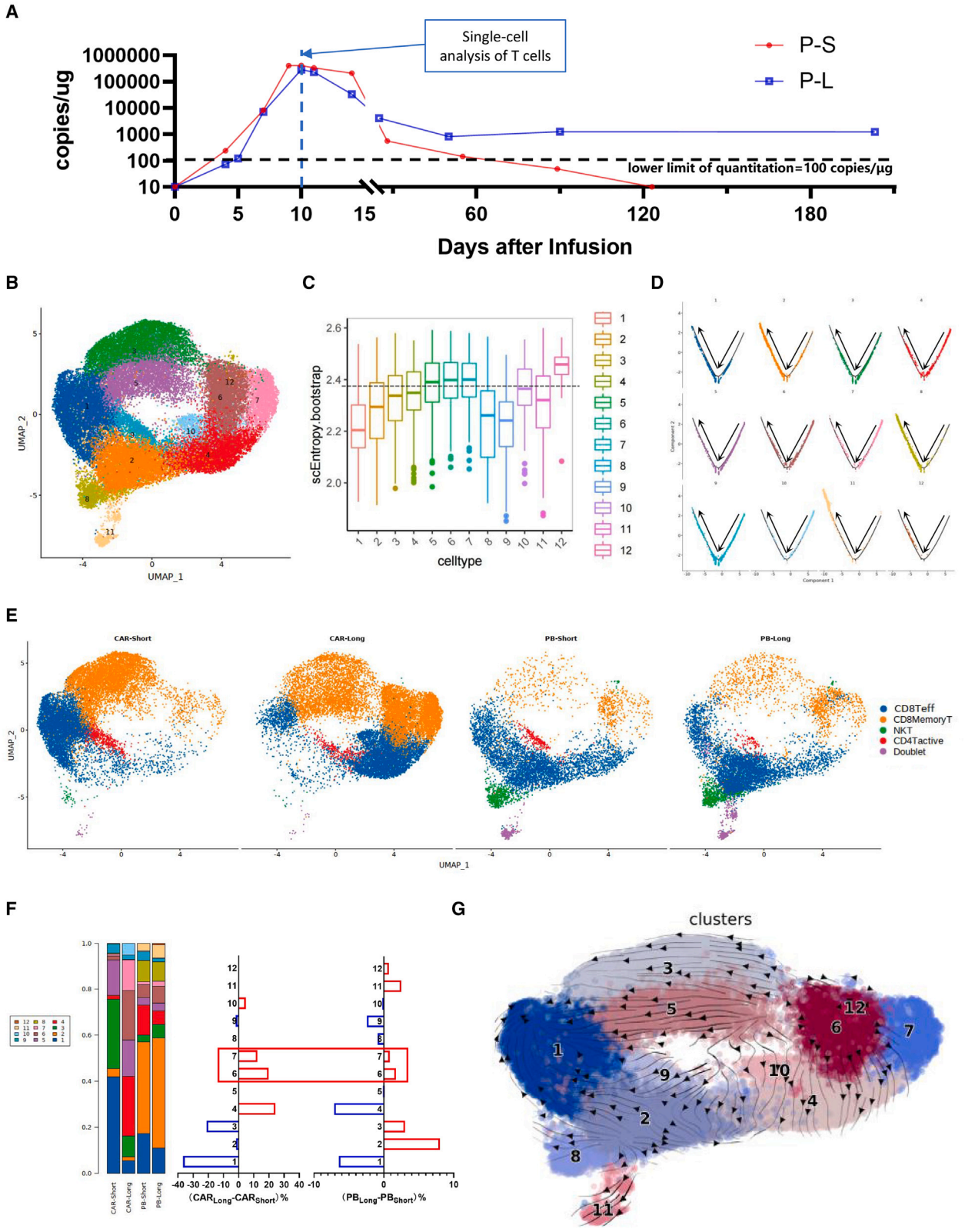
manufacture platform) with mild activation and shortened *in vitro* expansion time to about 2 days, exhibiting highest CAR-T potency and stemness phenotype. Then, we used the platform to produce CD19-targeted or CD19/CD22-dual-targeted CAR-T cells for 8 patients with B cell malignancies (3 with B cell lymphoma, and 5 with acute B cell lymphoblastic leukemia). The demographic and clinical characteristics of the patients and information of the infusion are shown in Table 3.

All 8 patients achieved CR after treatment, and no second-degree or higher cytokine release syndrome (CRS) and no neurotoxicity were observed. Loss of CAR-T cells was observed in only 1 patient, and the overall CAR-T persistence at 180 days was 80% (Figure 5A). B cells in PB of patients with CAR-T survival remained deficient, while P5 patients experienced B cell recovery due to loss of CAR-T (Figure 5B). The CAR copy numbers of PB of all 8 patients are shown in Figure 5C.

The above clinical result preliminarily suggested that the CAR-T cells produced by the PrimeCAR process have better potential for long-term persistence regardless of whether they are single target (CD19) or dual target (CD19/CD22). Further clinical research and long-term follow-up are still in progress.

The prime CAR-T shared common signaling pathways with the T-long subset

To verify whether PrimeCAR-T is closer to the T-long cell subset, we prepared conventional CAR-T and prime CAR-T cells from 6 donors' PBMCs, respectively, and analyzed the CAR-T cells using high-throughput RNA sequencing. GSVA of prime CAR-T and conventional CAR-T displays a similar differential pathway profile to T-long and T-short results. Prime CAR-T subsets also were more active in oxidative phosphorylation, MYC, E2F targets, NOTCH signaling, G2M checkpoint, MTORC1 signaling, glycolysis, fatty acid metabolism, DNA repair, and adipogenesis than conventional CAR-T (Figure 6A). We further used Pearson analysis to determine the signaling pathway correlation of PrimeCAR with T-long and T-other subsets, and we found that PrimeCAR was positively correlated with the T-long signaling pathway pattern and negatively correlated with the T-other signaling pathway pattern. In contrast, conventional CAR-T was positively correlated with the T-other signaling pathway pattern and negatively correlated with the T-long signaling pathway pattern (Figure 6B).



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DISCUSSION

Since the first report of successful clinical application of CD19-targeted CAR-T for lymphoma,[20] the therapeutic potential has been increasingly recognized and researched. In the treatment of r/r B-ALL, CD19-targeting CAR-T can often achieve a high CR rate without minimal residual disease (MRD); however, whether CAR-T therapy alone can be used as a curative treatment is still controversial. Currently most research reports are about bridging to transplantation after CAR-T treatment. From the independent studies of Huiwen Jiang et al. and Xian Zhang et al., it is not likely that long-term remission will be brought about by CD19-targeted CAR-T alone; however, the disease-free survival can be much improved if hematopoietic stem cell transplantation (HSCT) is bridged after the treatment.[21,22] The long-term follow-up done by Jae H. Park et al. showed that the 2 year disease-free survival rate of patients with ALL after CD19-targeted CAR-T was less than 20%, but their study pointed out that there was no significant difference in disease-free survival regardless of whether there was a bridging therapy or not after CAR-T treatment.[4] The need for bridging to transplantation after CAR-T treatment is indeed controversial; the study by Wei Chen et al. demonstrated that while bridging to transplantation after CAR-T therapy improved the disease-free survival rate, it also carried about 30% transplant-related deaths.[23] Therefore, it is of significance to improve the long-term disease-free survival of patients receiving CAR-T therapy alone through optimization of technology.

In addition to bridging transplantation after therapy, several studies have investigated other factors that may impact long-term prognosis after CAR-T therapy. The most frequently reported factor currently is tumor burden, which is inversely associated with long-term prognosis.[4,24,25] Some studies had shown that specific genetic abnormalities are associated with long-term prognosis.[22,26] In a study done by Kevin A. Hay, a prognostic analysis with 53 adult patients with ALL revealed that negative malignant clones validated by immunoglobulin heavy chain deep sequencing, low lactate dehydrogenase levels before preconditioning, high platelet levels before preconditioning, and a conditioning regimen with fludarabine contribute to a better prognosis.[5]

The team of Carl June et al. indicated that for patients that are not eligible for bridging to transplantation, a short persistence of CAR-T *in vivo* is unlikely to produce long-term remission.[16] Later, a global multi-center clinical study reported by this research team demonstrated that the long-term remission rate of CD19-targeted CAR-T in the treatment of children and adolescents with ALL can reach 59%, while only 9% of the patients were bridged to transplantation after the treatment. The median CAR-T persistence time reached 168 days, and most of the relapsed patients were CD19−.[27] Similar results were recently reported by Claire Roddie et al. in a study of CAR-T therapy for adult ALL.[28] Our findings not only demonstrated the importance of CAR persistence for long-term remission in patients with ALL but also the curative potential of CAR-T therapy alone in patients with CAR-T persistence *in vivo* for more than 6 months.

Our study shows that in the treatment of ALL, CD19/CD22-dual-targeted CAR-T does not show better long-term remission than CD19-targeted CAR-T. A study reported the median disease-free survival time for CAR-T treatment of ALL was only 5.8 months, while 10 out of 15 patients who responded to treatment experienced relapse.[17] We noticed that the CD19/CD22-dual-targeted CAR-T cells in our study did not persist for long *in vivo* in most patients, with unclear reasoning. We speculate that there may be interference between the single chain variable fragments (scFvs) of CD19 and CD22. It is also possible that the immunogenicity of CD22 caused an early rejection of CAR-T cells. In the case of assured persistence, whether CD19/CD22-dual-targeted CAR-T can better improve the long-term remission of patients than CD19-targeted CAR-T is a question worthy of future research.

The infused CAR-T cell products are heterogeneous, and this means that the long-term *in vivo* persistence of CAR-T may be related to some specific T cell subsets. Related research from memory T cells provides support for this concept.[29,30] A recent study that had followed up over 12 years showed that the long-persisted CAR-T cells in the body are a specific subgroup of CD4+ T cells.[31] We used single-cell sequencing technology to compare long-persisted and short-persisted CAR-T cells and determined a subset of T cells that may contribute to persistence. We will validate this result in future studies.

Previous studies have shown that shortening the time of *in vitro* culture can enhance the antitumor ability of CAR-T.[19,32] Our preliminary clinical results showed that CAR-T cells produced by shortening the time of *in vitro* culture have the potential for long-term *in vivo* persistence, with mild CRS and no immune effector cell associated neurotoxicity syndrome (ICANS). We will continue to carry out lymphatic research based on the PrimeCAR platform, including

Figure 3. Single-cell sequencing results

(A) CAR copy number results of P-S and P-L patients. CAR-T in P1 persisted for less than 2 months, and CAR-T in P2 persisted for more than 6 months. Both patients showed the peak of CAR-T amplification on the 10th day after infusion, and peripheral blood was taken for single-cell sequencing on the same day. (B) Single-cell sequencing was performed on the CAR-T cell products for infusion the PBMCs collected on the 10th day, clustering analysis of T cells and UMAP dimensionality reduction were displayed, and a total of 12 T cell subgroups were obtained. (C) The results of stemness analysis of 12 T cell subgroups. The top 4 stemness T cell subgroups were the 5th, 6th, 7th, and 12th. (D) The results of differentiation trajectory analysis of 12 T cell subsets. The top right is the initial stage of differentiation, and the top left is the final stage of differentiation. The 4th, 5th, 6th, 7th, and 10th cell subgroups were mostly concentrated in the early stage of differentiation. (E) The result of T cell annotation. The 3rd, 5th, 6th, and 7th clusters are recognized as CD8+ memory T cells. (F) The proportion of each T cell subgroups in the 4 specimens. The horizontal bar graph presents the difference of proportion of each specific T cell subgroups in the four samples tested, among which the 6th and 7th clusters are more abundant in the long-term persistent samples than in the short-term persistent samples. (G) The differentiation trajectory of the 12 clusters of T cells.

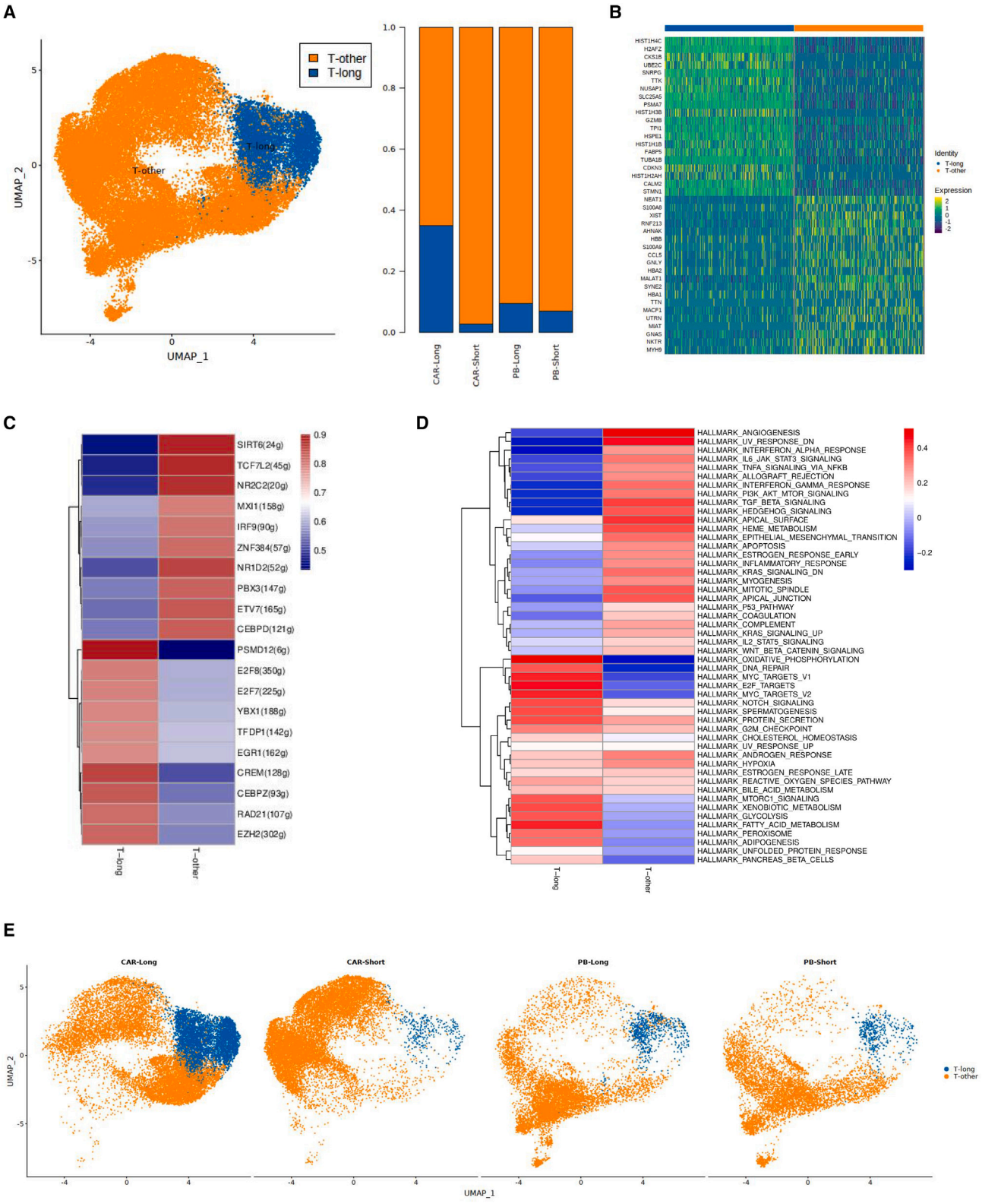

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Table 3. Characteristics of the patients treated with prime CAR-T

No.	Gender	Age (year)	Disease	High-risk factor	CAR-T target	Transplantation history	CAR-T dose, ◇10^6/kg	CRS grade	Best response
P1	male	64	NHL	–	CD19	–	0.24	0	CR
P2	male	75	NHL	–	CD19	auto-HSCT	0.48	0	CR
P3	female	9	ALL	MLL-AF4	CD19+CD22	–	0.18	1	CR
P4	female	5	ALL	–	CD19+CD22	–	0.33	1	CR
P5	female	12	ALL	MEF2D-BCL9CDKN2A/B-	CD19+CD22	allo-HSCT	1.95	1	CR
P6	female	55	ALL	–	CD19+CD22	–	0.2	1	CR
P7	female	19	ALL	–	CD19+CD22	–	0.17	1	CR
P8	male	52	NHL	–	CD19+CD22	–	0.44	1	CR

NHL, non-Hodgkin's lymphoma; auto-HSCT, autologous hematopoietic stem cell transplantation; allo-HSCT, allogeneic hematopoietic stem cell transplantation.

CD19-targeted and CD19/CD22-dual-targeted CAR-T with the expectation that the PrimeCAR platform can promote CAR-T therapy into a better curative intervention.

MATERIALS AND METHODS

Study description

The data analysis in this article came from three clinical studies: ClinicalTrials.gov: NCT02349698, NCT04271410, and NCT04649983. The ClinicalTrials.gov: NCT02349698 project was carried out at Southwest Hospital of the Third Military Medical University. The cut-off time of data was December 31, 2021. The purpose of the research is to verify the safety and efficacy of CD19-targeted CAR-T in B cell leukemia and lymphoma. Both ClinicalTrials.gov: NCT04271410 and NCT04649983 were carried out at the 920th Hospital of Joint Logistics Support Force of the Chinese People's Liberation Army. The purpose of that research is to verify the safety and efficacy of CD19-targeted CAR-T or CD19/CD22-dual-targeted CAR-T in the treatment of B cell malignancy. The clinical studies were approved by the ethics committee of each institution. Each clinical study was designed in accordance with the Declaration of Helsinki. Study descriptions and details can be found on the clinicaltrials.gov website. The data screening process is presented in Figure 1A.

The methodology of CD-19-targeted CAR-T cell production, flow cytometry, and PCR molecular screening was published.[14,15] The CAR-T structure of CD19/CD22 is a tandem scFv structure followed by CD8 hinge and transmembrane domain, CD137 costimulatory domain, and the signaling portion of CD3 ζ. PrimeCAR is our new cell production platform based on the principle of mild activation and shortening of *in vitro* culture process. In which, T cells were enriched from leukapheresis, followed by activation and transduction with a lentiviral vector encoding for the CAR molecule. After about 24 h of culture, cells were harvested.

Demographic characteristics

A total of 50 ALL subjects were included in the analysis, of which 35 were treated with CD19 single-target CAR-T (CD19 group) and 15 were treated with CD19/CD22-dual-target CAR-T (CD19/CD22 group) without receiving allo-stem cell transplantation before relapse after CAR-T therapy (Table 1). Fifteen patients who received CD19 single-target CAR-T therapy were from the ClinicalTrials.gov: NCT02349698 project and were reported in the previous studies.[14,15] There is no significant difference between the CD19 group and the CD19/CD22 group in gender, age, infusion dosage, allogeneic transplantation history, baseline burden, cytogenetic factor, CNS involvement, or extramedullary lesions (supplemental materials and methods).

CAR-T culture

The ordinary CAR-T cultivation is reported in our previous report.[15] For the short-period PrimeCART cell culture method, T cells were purified from the leukapheresis material of donors by CD4 isolation kit (170-076-702, Miltenyi Biotec) and CD8 isolation kit (170-076-703, Miltenyi Biotec) according to the instructions. After isolation, T cells were activated by 50 ng/mL anti-CD3 antibody (170-076-116, Miltenyi Biotec) on day 0. T cells were then transduced with lentivirus on day 1 and expanded for less than 24 h in the medium supplemented with 5% autologous plasma serum, 5 ng/mL interleukin-7 (IL-7; 200–07, Peprotech), and 25 ng/mL IL-21 (200–21, Peprotech). Viability and concentration were determined by the automated cell counter (Rigel S2, Countstars) every other day.

Figure 4. Results of in-depth analysis of 6^{th} and 7^{th} T cell clusters

(A) The 6^{th} and 7^{th} cell subgroups were merged into one group, named T-long. The remaining 10 cell subgroups were merged into another group, named T-other. The UMAP map and the proportion of two cell subgroups in CAR-T product were obtained again. (B) The heatmap of the top 10 genes expressed in T-long and T-short cell subgroups. (C) The heatmap of transcriptional regulation analysis of T-long and T-short cell subgroups. The parentheses next to the transcription factor names indicate the number of genes that were detected to be regulated by that transcription factor in the specimen, and the color indicates the intensity of enrichment. (D) The GSVA analysis of T-long and T-other cell subgroups, color represents activity. The T-long cell subgroup are more active in MYC and E2F targets, oxidative phosphorylation, glycolysis, fatty acid metabolism, DNA repair, and adipogenesis pathways. (F) The distribution of T-long and T-short cells in the four samples.

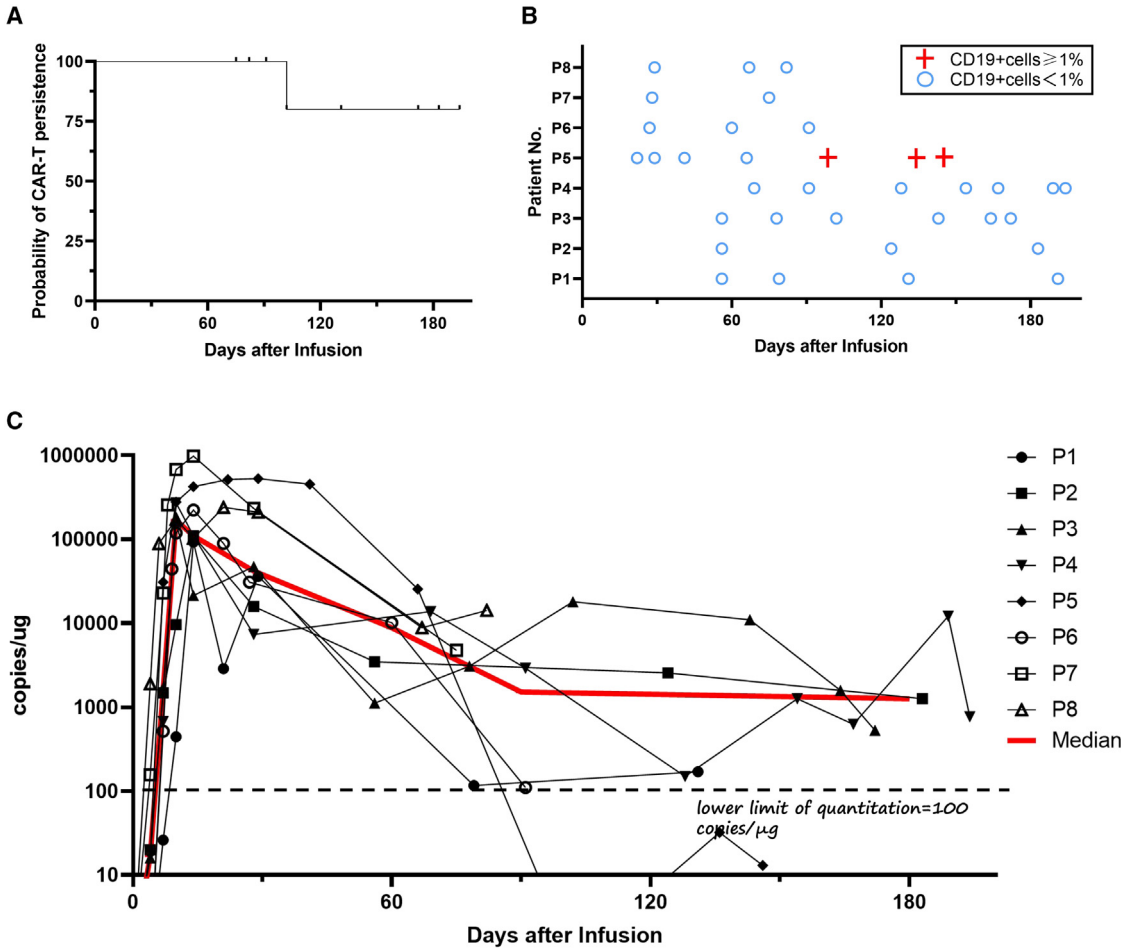

Figure 5. Prime CAR-T preliminarily shows potential for long-term persistence

(A) The Kaplan-Meier curve of PrimeCAR-T cells persisting in peripheral blood of patients. The persistence rate at day 180 is 80%. (B) The screening results of CD19+ target cells in the peripheral blood of patients. Only the P5 patients had recovery of CD19+ cells, and the other patients maintained the state of CD19+ cell absence. (C) The CAR copy-number detection results in peripheral blood of patients. CAR copy number <100 was considered the absence of CAR-T cells. Of all 8 patients who received PrimeCAR-T cell infusion, only P5 patients experienced CAR-T cell loss.

Statistical analysis

The baseline comparison for categorical variable was done by the chi-squared test or the Fisher's exact test. Pearson analysis was used to determine the signaling pathway correlation of PrimeCAR with T-long and T-other subsets. The Student's t test was used to assess baseline difference between for numeric variables. Univariate risk analysis was computed by Cox regression, and the risk was presented by hazard ratio (HR). The EFS was estimated by using the Kaplan-Meier method, and difference was assessed with the log rank test by GraphPad Prism 5 software. A p value of <0.05 was considered statistically significant.

Single-cell sequencing

For the single-cell sequencing, the workflow is shown in Figure 1B. The single-cell sequencing work was done by Singleron Biotechnologies in Nanjing. For more specific methodology, please refer to the supplemental materials and methods.

DATA AVAILABILITY

Some data, models, and code generated or used during the study are available from the corresponding author by request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.04.003.

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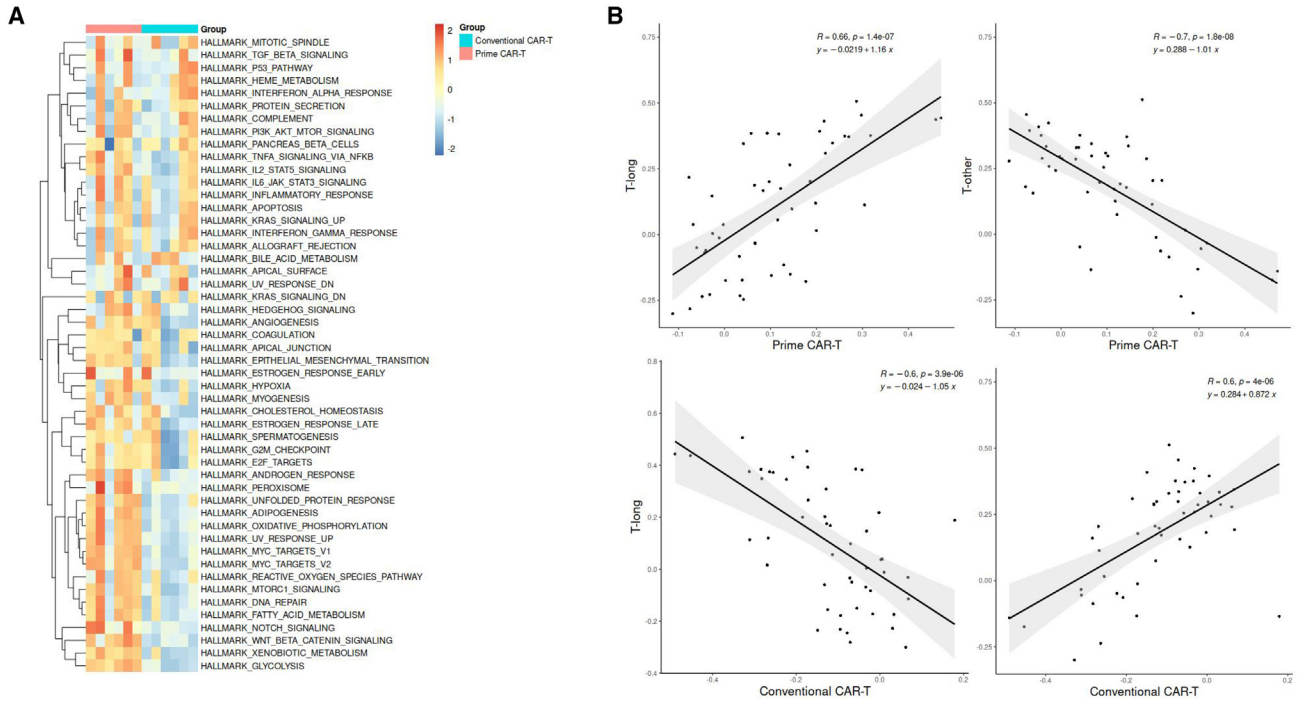

Figure 6. The relation analysis among the T-long subset, the T-other subset, the PrimeCAR-T, and the conventional CAR-T

(A) The prime CAR-T and conventional CAR-T display a similar differential pathway profile to T-long and T-short subsets. (B) The relation of signaling pathways among the T-long subset, the T-other subset, the PrimeCAR-T, and the conventional CAR-T.

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AUTHOR CONTRIBUTIONS

W.S., Y. Zhi, and Q.C. designed the study. L.S. wrote the manuscript. L.S., Z.J., L. Lin, Y. Zhongtao, Chen Zucong, Li Yu, L. Le, F.L., C.Y., W.Y., Z.D., L.X., and D.Y. provided clinical support. X.H. performed bioinformatics analysis. C.L., Z.Q., S.J., Z.Y., L. Yunyan, W.L., W.M., Z.W., and Y.P. performed the *in vitro* experiment. L.S., H.L., H.X., Z.L., and Z.Z. analyzed data. Y. Zhi, W.S., and Q.C. reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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